Differential Release of Cellular and Scrapie Prion Proteins from Cellular Membranes by Phosphatidylinositol-Specific Phospholipase C†

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ABSTRACT: The abnormal isoform of the scrapie prion protein PrP^{Sc} is both a host-derived protein and a component of the infectious agent causing scrapie. PrP^{Sc} and the normal cellular isoform PrP^{C} have different physical properties that apparently arise from a posttranslational event. Both PrP isoforms are covalently modified at the carboxy terminus by a glycoinositol phospholipid. Using preparations of dissociated cells derived from normal and scrapie-infected hamster brain tissue, we find that the majority of PrP^{C} is released from membranes by phosphatidylinositol-specific phospholipase C (PIPLC), while PrP^{Sc} is resistant to release. In contrast, purified denatured PrP 27–30 (which is formed from PrP^{Sc} during purification by proteolysis of the amino terminus) is completely cleaved by PIPLC. Incubation of the cell preparations with proteinase K cleaves PrP^{Sc} to form PrP 27–30, demonstrating that PrP^{Sc} is accessible to added enzymes. We have also developed a protocol involving biotinylation that gives a quantitative estimate of the fraction of a protein exposed to the cell exterior. Using this strategy, we find that a large portion of PrP^{Sc} in the cell preparations reacts with a membrane-impermeant biotinylation reagent. Whether alternative membrane anchoring of PrP^{Sc}, inaccessibility of the glycoinositol phospholipid anchor to PIPLC, or binding to another cellular component is responsible for the differential release of prion proteins from cells remains to be determined.

Scrapie is a transmissible neurodegenerative disease caused by an infectious agent called a prion because of its unusual properties (Prusiner, 1982, 1989). The scrapie prion is extremely small, about 55 kDa as calculated from its sensitivity to inactivation by ionizing radiation (Bellinger-Kawahara et al., 1988). Scrapie prion infectivity is extraordinarily resistant to procedures that modify or destroy nucleic acids (Alper et al., 1967; Hunter, 1979; Prusiner, 1982; Diener et al., 1982; McKinley et al., 1983b; Bellinger-Kawahara et al., 1987a,b; Gabizon et al., 1987, 1988a). Highly purified infectious fractions contain a protease-resistant glycoprotein called PrP 27–30 (Bolton et al., 1982, 1984; McKinley et al., 1983a; Prusiner et al., 1982, 1983; Diringer et al., 1983), which is derived from a larger protein (PrP^{Sc})[1] during purification by limited proteolysis at the amino terminus (Prusiner et al., 1984; Oesch et al., 1985; Meyer et al., 1986; Barry & Prusiner, 1986; Hope et al., 1986). We have concluded that this protein is a prion component because all attempts to separate scrapie infectivity from PrP 27–30 or PrP^{Sc} have failed (Prusiner et al., 1982, 1983, 1984; Gabizon et al., 1987; Manuelidis et al., 1987). Furthermore, the titer of scrapie infectivity correlates with the amount of PrP 27–30 remaining after an extended proteinase K digest (McKinley et al., 1983b). Scrapie infectivity is also specifically recovered from a monoclonal anti-PrP immunoaffinity matrix (Gabizon et al., 1988b).

PrP^{Sc} is related to a normal cellular protein of unknown function called PrP^{C} (Oesch et al., 1985; Barry et al., 1986; Cho, 1986; Turk et al., 1988; Bendheim et al., 1988). The two PrP isoforms have different physical properties. PrP^{C} is rapidly degraded by proteases and is soluble in the presence of detergents (Oesch et al., 1985; Meyer et al., 1986); PrP^{Sc} gives PrP 27–30 upon proteolysis, and PrP 27–30 polymerizes in the presence of detergents to form amyloid rods (Barry et al., 1985; Meyer et al., 1986; McKinley et al., 1988). All of the available evidence indicates that PrP^{Sc} and PrP^{C} have the same primary amino acid sequence and differ by some posttranslational event (Oesch et al., 1985; Basler et al., 1986; Borchelt et al., 1990). Although the presence of additional prion components such as a small nucleic acid has not been ruled out, the possibility remains that the infectious particle consists of only PrP^{Sc}.

We have begun to catalog and compare the posttranslational modifications of PrP^{Sc} and PrP^{C} to determine whether chemical differences account for the distinct physical properties that distinguish these two isoforms. Although no chemical differences between the two isoforms have yet been reported, several candidate structures exist. Both isoforms contain two N-linked oligosaccharides that are resistant to Endo H (Bolton et al., 1985; Meyer et al., 1986; Haraguchi et al., 1989); the structures of these sugars are known for PrP^{Sc} (Endo et al., 1989). There appear to be two arginine residues with unknown modifications at positions 3 and 15 of PrP^{Sc} (Hope et al., 1988; Turk et al., 1988); a missing cycle at Arg-3 is also observed in the Edman degradation of the first 10 residues of PrP^{C} (Turk et al., 1988). Both PrP^{C} and PrP^{Sc} also contain a glycoinositol phospholipid (abbreviated GPI) attached at the C-terminus (Stahl et al., 1987). The majority of PrP^{C} is released from the surface of cultured cells with *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PIPLC) (Stahl et al., 1987), which cleaves diradylglycerol

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[1] Abbreviations: PrP^{Sc}, scrapie isoform of the scrapie prion protein; PrP^{C}, cellular isoform of the scrapie prion protein; GPI, glycoinositol phospholipid; PIPLC, phosphatidylinositol-specific phospholipase C; CRD, cross-reacting determinant; TFA, trifluoroacetic acid; VSG, variant surface glycoprotein; NHS-SS-biotin, 3-sulfosuccinimido 3-[[2-(biotinamido)ethyl]dithio]propionate; PMSF, phenylmethanesulfonyl fluoride.

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from the glycolipid (Low, 1987; Ferguson & Williams, 1988). Purified PrP 27–30 is also at least partially susceptible to cleavage by PIPLC (Stahl et al., 1987).

We report here that PrP^{C} and PrP^{Sc} differ in their releasibility from cellular membranes with PIPLC. PrP^{C} is largely released from cells derived from both normal and scrapie-infected hamsters, while PrP^{Sc} remains bound to the cell. However, purified, denatured PrP 27–30 is completely susceptible to PIPLC. PrP^{Sc} in these membranes is accessible to both biotinylation and proteolysis of the amino terminus by proteinase K, demonstrating that total inaccessibility to the phospholipase does not account for the lack of release.

Experimental Procedures

Analytical quality reagents were used throughout. Iodobeads and NHS-SS-biotin were purchased from Pierce (Rockford, IL). Endoproteinase Lys-C (sequencing grade) and streptavidin–agarose were obtained from Boehringer Mannheim (Indianapolis, IN) and Sigma (St. Louis, MO), respectively. Water for HPLC was purified with a Millipore (Bedford, MA) Milli-Q system. The anti-tubulin monoclonal antibody was from ICN (Irvine, CA), while the anti-PrP antibodies used have been described previously (Barry & Prusiner, 1986; Barry et al., 1988). The affinity-purified cross-reacting determinant (CRD) antiserum was a generous gift from Judy Fox, and *Bacillus thuringiensis* PIPLC was a gift from Martin Low.

Preparation of Dissociated Cells. Dissociated brain cells were prepared from clinically sick scrapie-infected hamsters and age-matched uninfected hamsters with modifications of the methods of Chao and Rumsby (1977) and Norton and Poduslo (1970). Animals were anesthetized in a CO_2 chamber prior to cervical dislocation followed by immediate removal of the brain. The brain was placed in a Petri dish on ice and the cerebellum discarded. The remainder of the brain was bathed in HAP medium (5% glucose, 5% fructose, and 1% BSA in 100 mM KH_2PO_4–NaOH buffer at pH 6.0; 5 mL per brain) and minced with a razor blade. Minced chunks from three brains were digested with 1 unit of Collagenase in 10 mL (UCSF Cell Culture Facility) for 90 min at 37 °C with gentle mixing. Following digestion, an equal volume of cold, buffered fetal calf serum was added to the reaction mixture, and it was held on ice for 5 min. Sedimentation of cell and tissue pieces was at 1000 rpm for 5 min at 4 °C in a Beckman J-6 centrifuge.

The 1000 rpm cell pellet was washed twice with HAP medium containing BSA and sedimented in the J-6 at 3000 rpm for 5 min. These cell chunks were sieved sequentially through a 96-μm nylon mesh (once) and a 74-μm steel mesh (twice) into a Büchner funnel with light vacuum suction. Sieved cells were separated into specific types in a discontinuous sucrose gradient containing, from bottom to top, 1.5 mL of 2.0 M sucrose (in HAP–BSA medium), 1.5 mL of 1.55 M sucrose, 1.5 mL of 1.35 M sucrose, 5.0 mL of 0.9 M sucrose, and 3.0 mL of sample. These gradients were centrifuged at 4390 rpm for 10 min in an SW 41 rotor. Neuronal cells were collected at the 1.55/2.0 M interface, and glial cells were collected at the 0.9/1.35 M interface (Chao & Rumsby, 1977). The sucrose solutions were diluted slowly with cold PBS, and the cells were then sedimented out of the sucrose by centrifugation at 3000 rpm, 15 min at 4 °C in a J-6.

Biotinylation Assay. The isolated cells were suspended on ice for 15 min in PBS containing 1 mg of the amino-reactive compound NHS-SS-biotin per milliliter and then collected by centrifugation at 1000*g* for 3 min and washed once with TBS to quench any unreacted NHS-SS-biotin. Cellular proteins were extracted in TBS with 0.5% each of deoxycholate and NP-40 and then precipitated with 4–10 volumes of ethanol at −20 °C. Approximately 3 μg of rabbit immunoglobulin, which had previously been reacted with NHS-SS-biotin, was added as an internal standard. Each sample was then boiled in 0.5 mL of TBS containing 2% SDS to denature the proteins and prevent nonspecific absorption. Streptavidin–agarose was washed twice with TBS containing 2% SDS and 1 mM PMSF. Biotinylated proteins were then bound batchwise onto 200 μL of streptavidin–agarose, and the unbound fraction in the supernatant was combined with a single wash of the streptavidin–agarose beads. Bound proteins were released from the beads by incubation for 20 min in TBS supplemented with 10% 2-mercaptoethanol. The two fractions were then examined by immunoblotting for their content of PrP; the bound portion represents the fraction of protein accessible to the membrane-impermeable reagent.

Iodination and Endoproteinase Lys-C Digestion of PrP 27–30. PrP 27–30 was purified and precipitated from sucrose gradient fractions as described previously (Stahl et al., 1987). The protein pellet was denatured and solubilized by the addition of 6 M guanidine hydrochloride containing 50 mM Tris hydrochloride (pH 8.45) and 5 mM EDTA. The protein was reduced by the addition of dithiothreitol (2 mM final concentration) for 1 h at room temperature and carboxymethylated with 6 mM sodium iodoacetate at 4 °C for 1.5 h. The guanidine hydrochloride was removed by precipitation of the reduced and carboxymethylated PrP 27–30 with ≥10 volumes of ethanol at −20 °C for ≥2 h. The protein pellet was washed with ice-cold ethanol to remove the last trace of guanidine hydrochloride, suspended in 50 mM Tris-HCl (pH 8.45) containing 0.1% SDS, and iodinated with Iodobeads (Pierce) according to the manufacturer's suggested procedure. Unincorporated [^{125}I]iodide was removed by the ethanol precipitation and washing procedure described above. Radioiodinated PrP 27–30 was resuspended in 50 mM Tris-HCl (pH 8.45) containing 0.1% SDS and 1 mM EDTA and then digested with ~1 μg of endoproteinase Lys-C at 37 °C.

HPLC. Radioiodinated peptides derived from PrP 27–30 were separated by reverse-phase chromatography on a Vydac C4 column with a Gilson HPLC system. The solvents used were A = 0.1% TFA and B = 80% acetonitrile with 0.1% TFA. Peptides were eluted with a gradient consisting of 0% B for 10 min, 0–50% B at 60 min, and then 100% B at 67 min, at a flow rate of 0.75 mL/min. Fractions were collected at 1-min intervals, and their content of ^{125}I was determined with a Beckman γ-counter.

Electrophoresis and Autoradiography. SDS–PAGE was carried out either according to Laemmli (1970) or with the tricine buffer system described by Schägger and von Jagow (1987). Protein standards for these electrophoresis systems were the low molecular weight standards from Bio-Rad (Richmond, CA) or the MW-SDS-17 standard from Sigma, respectively. With the tricine buffer system, the gels consisted of three zones with 4%, 10%, and 16% acrylamide as described (Schägger & von Jagow, 1987). To avoid the loss of very small peptides, the tricine gels were not fixed or stained before autoradiography at −70 °C with an intensifying screen. The one exception is the autoradiograph in Figure 6A, which was obtained after the gel was soaked in 50% methanol/10% trichloroacetic acid and then cross-linked in 10% formaldehyde before being stained with Coomassie blue to visualize the protein standards.

Results

We chose to use preparations of dissociated cells derived

from the brains of adult normal and scrapie-infected hamsters for these experiments for several reasons. (1) These cells contain far more PrP^{Sc} than scrapie-infected cells in culture (Butler et al., 1988) and provide a benchmark for comparison to similar experiments carried out with transformed cells, which are reported elsewhere (Borchelt et al., 1990). (2) Initial experiments with brain microsomal preparations required high-speed centrifugation at 100000*g* to separate PIPLC-released and membrane-bound proteins, while experiments on the dissociated cell preparations only require centrifugation at 1000*g*. Low-speed centrifugation at 1000*g* is advantageous because aggregation of PrP^{Sc} would cause it to pellet after centrifugation at 100000*g*, thus obscuring whether it was released by PIPLC. (3) Although studies on scrapie-infected cultures of mouse neuroblastoma N_2a cells indicate that a substantial fraction of the PrP^{Sc} is localized to an internal compartment (Taraboulos et al., 1990), the results shown below indicate that the disrupted cell preparations are leaky and a majority of PrP^{Sc} is accessible to both enzymatic digestion by proteinase K and biotinylation by a membrane-impermeant reagent.

The brain cells used in this study were prepared by a modification of a method used for isolation of cells from neonatal rodent brains (Norton & Poduslo, 1970; Chao & Rumsby, 1977), as described under Experimental Procedures. Preliminary experiments indicated that cell disruption with trypsin resulted in the hydrolysis of PrP^{C}. However, we found that collagenase appears to give adequate cell disruption and leaves both PrP^{C} and PrP^{Sc} intact. Centrifugation of the disrupted cells on sucrose gradients yields fractions enriched for either neuronal or glial cell markers (not shown); both fractions gave identical results unless otherwise indicated.

Differential Release of PrP^{Sc} and PrP^{C}. The immunoblot in Figure 1A demonstrates the difference in the releasibility of PrP^{C} and PrP^{Sc} by PIPLC. Incubation of normal cells with PIPLC for 3 h results in a marked release of immunoreactive PrP^{C} into the supernatant relative to that observed in the absence of PIPLC (Figure 1A, lane 4 vs lane 2). Cells derived from scrapie-infected brains also release immunoreactive protein upon incubation with PIPLC (Figure 1A, lane 8 vs lane 6), but a substantial fraction of the immunostaining remains in the cell pellet after centrifugation (Figure 1A, lane 7). Scrapie-infected cells contain both PrP^{Sc} and PrP^{C}, which can be distinguished by digestion with proteinase K. PrP^{C} is sensitive to proteinase K, while PrP^{Sc} loses only its amino terminus to give a protease-resistant core designated PrP 27–30. Digestion of the samples with proteinase K reveals that all of the protease-resistant protein (PrP^{Sc}) remains in the cell pellet (Figure 1A, lower panel, lane 7), while all of the protein released into the supernatant is sensitive to proteinase K, diagnostic for PrP^{C} (Figure 1A, lower panel, lane 8). We have observed identical results with cell fractions enriched for neurons or glia derived from hamsters sacrificed either 65 or 45 days after intracerebral inoculation with scrapie prions. At 45 days after inoculation high titers of prions and substantial levels of PrP^{Sc} are found in brain, but there is virtually no detectable neuropathology (Baringer et al., 1981). By 65 days, extensive neuronal degeneration is found.

PrP^{Sc} and PrP 27–30 can aggregate upon detergent extraction (Meyer et al., 1986; McKinley et al., 1988). Although PrP^{Sc} behaves as if it is functionally solubilized in the presence of lipid membranes (Gabizon et al., 1987), it is conceivable that the protein could aggregate after PIPLC treatment and sediment at 1000*g*. We tested this possibility by adding purified PrP 27–30, which is aggregated in amyloid rods, to a

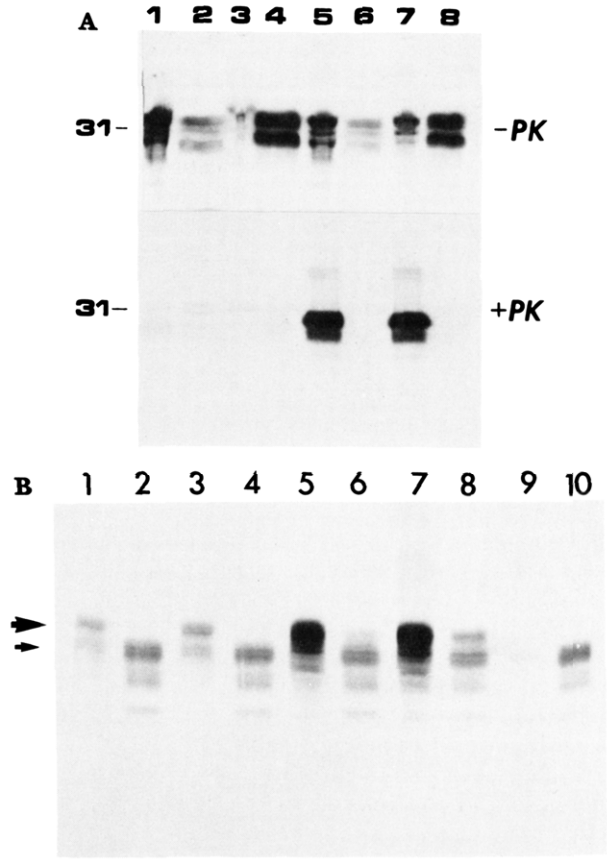

FIGURE 1: Differential release of PrP^{C} and PrP^{Sc} from dissociated cells. (A) Glial-enriched dissociated cells from normal (lanes 1–4) or scrapie-infected (lanes 5–8) hamsters were incubated in PBS (lanes 1, 2, 5, and 6) or PBS + 0.3 unit of PIPLC (lanes 3, 4, 7, and 8) at room temperature for 3 h with gentle shaking. Cell-associated (odd lanes) and supernatant fractions (even lanes) were separated by centrifugation at 1000*g* for 3 min. Proteins were extracted from the cell pellet as described under Experimental Procedures; this extract and the supernatant fraction were then precipitated with 4–10 volumes of ethanol at −20 °C, subjected to SDS–PAGE in 12% acrylamide gels, and immunoblotted with monoclonal antibody 13A5. For the bottom panel, the cell pellet extract and the supernatant fraction were digested with 50 μg/mL proteinase K at 37 °C for 30 min before the addition of 1 mM PMSF and precipitation with ethanol. (B) Purified PrP 27–30 in amyloid rods was incubated with neuron-rich cells (lanes 1–4), glial-rich cells (lanes 5–8), or PBS only (lanes 9 and 10) for 30 min at room temperature. Cell-associated (odd lanes) and supernatant fractions (even lanes) were isolated as described above and immunoblotted. The large arrow indicates immunostaining of PrP molecules of 33–35 kDa, and the small arrow shows immunostaining of PrP 27–30.

suspension of cells derived from normal or scrapie-infected hamster brain (Figure 1B). After centrifugation at 1000*g*, immunoblots of the cell pellets (Figure 1B, odd lanes) and supernatant fractions (Figure 1B, even lanes) indicate that the added PrP 27–30, which migrates faster than PrP^{Sc} or PrP^{C} on SDS–PAGE, is found primarily in the supernatant fractions. Identical results are obtained regardless of whether the neuron-rich cell fraction (lanes 1–4) or glial-rich cell fraction (lanes 5–8) is examined. PrP 27–30 amyloid rods also remain in the supernatant fraction if centrifuged in the absence of cells (lanes 9 and 10). This result suggests that if similar aggregation of PrP^{Sc} occurred upon release, PrP^{Sc} would still be found in the supernatant after centrifugation.

Accessibility of PrP^{Sc} to Protease. The accessibility of cell-associated PrP^{Sc} to enzymes was probed by incubation of proteinase K with normal and scrapie cell preparations. Following inhibition of protease activity with PMSF, the cells were collected by centrifugation to remove the protease, and

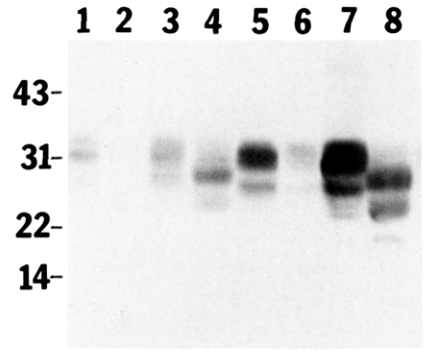

FIGURE 2: Proteinase K accessibility of PrP^C and PrP^{Sc} in dissociated cells. The neuron-rich cell fraction (lanes 1–4) and glial-rich cell fraction (lanes 5–8) from normal (lanes 1, 2, 5, and 6) or scrapie-infected hamsters (lanes 3, 4, 7, and 8) were incubated in PBS without (odd lanes) or with (even lanes) 20 μg/mL proteinase K at room temperature for 30 min. Following addition of 1 mM PMSF, the cells were collected by centrifugation in a microfuge. Protein was extracted from the cell pellets and precipitated with ethanol. The figure shows an immunoblot of the samples probed with monoclonal antibody 13A5.

cellular proteins were extracted for immunoblotting. Cell preparations from normal brain show almost complete hydrolysis of PrP^C by the protease, consistent with its cell surface localization and sensitivity to PIPLC (Figure 2, lanes 2 and 6). Immunoblots of proteinase K treated cells from scrapie-infected brain show that the majority of PrP^{Sc} has lost its amino terminus to form PrP 27–30, as indicated by the decrease in molecular weight (Figure 2, lanes 4 and 8). Identical results were obtained when small chunks of minced brain tissue, as opposed to isolated cells, were treated with proteinase K (not shown). The susceptibility of PrP^{Sc} to conversion into PrP 27–30 by limited digestion of cells with proteinase K demonstrates that PrP^{Sc} is accessible to exogenously added protease. This finding suggests that PrP^{Sc} is probably also accessible to PIPLC. Because the dissociated brain cells possessed damaged plasma membranes, the susceptibility of PrP^{Sc} to limited proteolysis does not permit assignment of subcellular localization. The biotinylation studies described below are consistent with these conclusions.

Cell Surface Biotinylation. Because of the possibility that proteinase K might in some way create holes in the cell membrane and expose PrP^{Sc}, we decided to pursue an independent verification of the accessibility of PrP^{Sc}. Another widely used method for probing cell surface localization of proteins is the labeling of intact cells by iodination, followed by immunoprecipitation. Although this procedure is sensitive, it suffers from the inability to quantitatively determine what fraction of the protein is exposed on the cell surface. To supplement this method, we have developed a biotinylation procedure to quantitatively estimate what fraction of a molecule is exposed on the cell surface. Following biotinylation of cells, the accessible fraction of a protein is that which specifically binds to streptavidin–agarose (see Experimental Procedures).

The results of this procedure are shown in Figure 3. Neither PrP^C (panel A, lanes 1 and 2), PrP^{Sc} (panel A, lanes 5 and 6), nor PrP 27–30 (panel B, lanes 5 and 6) binds streptavidin–agarose when the cells are not biotinylated. In contrast, the biotinylated rabbit IgG added as an internal standard is quantitatively bound. Upon biotinylation, more than half of the PrP^C appears in the bound fraction (panel A, lanes 3 and 4). However, only half of the biotinylated IgG is retained in this bound fraction, indicating that insufficient streptavidin–agarose was used to bind all of the biotinylated

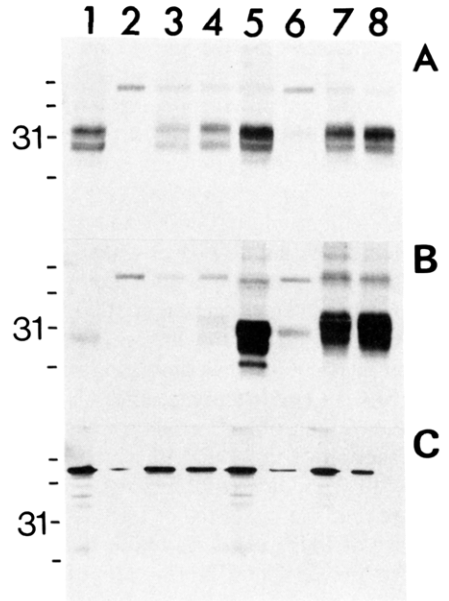

FIGURE 3: Cell surface biotinylation of PrP in dissociated cells. Glial-enriched dissociated cells from normal (lanes 1–4) or scrapie-infected (lanes 5–8) hamsters were biotinylated (lanes 3, 4, 7, and 8) or not biotinylated (lanes 1, 2, 5, and 6), half the sample was digested with proteinase K (panel B), and both were carried through the protocol described under Experimental Procedures. Following fractionation on streptavidin–agarose, the unbound (odd lanes) and bound fractions (even lanes) were subjected to immunoblotting with a rabbit polyclonal antibody raised against a synthetic PrP peptide (panels A and B) or an anti-tubulin monoclonal antibody (panel C). The molecular mass markers indicate 65, 45, 31, and 21.5 kDa, respectively. The band observed at 55 kDa in panels A and B is biotinylated rabbit IgG added as an internal standard as described under Experimental Procedures.

proteins. Similarly, about half of the protease-resistant PrP 27–30 appears in the bound fraction (panels A and B, lanes 7 and 8). Since approximately the same amount of the internal standard is also bound, this result indicates that the majority of PrP^C and PrP^{Sc} are accessible to biotinylation by the membrane-impermeant reagent.

We also examined the biotinylation of tubulin to determine the extent of leakiness of the cell preparations (Figure 3C). A significant fraction of the tubulin is observed in the bound fractions in both the normal and scrapie cell preparations (lanes 4 and 8). This indicates that a majority of the cells are not intact.

PIPLC Release of Other Glycolipidated Proteins. We investigated the ability of PIPLC to release other glycolipidated proteins from normal and scrapie-infected cells using an affinity-purified CRD antiserum that is directed against an epitope on the GPI anchor (also known as the cross-reactive determinant or CRD) of the variant surface glycoprotein (VSG) of trypanosomes (Barbet & McGuire, 1978; Cross, 1979; Holder & Cross, 1981). This CRD antiserum binds to VSG only after PIPLC cleavage and cross-reacts with many mammalian GPI-anchored proteins (Stieger et al., 1986; Davitz et al., 1987a; Stahl et al., 1987; Ferguson & Williams, 1988). PIPLC-released supernatants from both normal and scrapie-infected cells contain comparable amounts of a variety of anti-CRD reactive proteins (Figure 4, lanes 4 and 8). No cross-reactive bands are observed in the absence of PIPLC cleavage (lanes 2 and 6). Interestingly, we observed no anti-CRD reactive bands in the neuron-rich fraction (not shown). This suggests that the structures of the GPI modifications differ in the two cell types in a way that makes the neuronal proteins unreactive to anti-CRD. A second possibility is that there are much lower amounts of GPI-anchored proteins

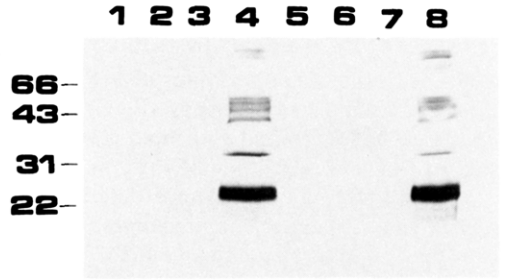

FIGURE 4: Anti-CRD reactivity of proteins released from dissociated cells by PIPLC. Dissociated cells from normal (lanes 1–4) or scrapie-infected (lanes 5–8) hamsters were incubated with PBS (lanes 1, 2, 5, and 6) or PBS + 0.3 unit of PIPLC (lanes 3, 4, 7, and 8). Samples were centrifuged at 1000g to recover the cell pellet (odd lanes) or supernatant (even lanes) fractions. Proteins were extracted from the cell pellet as described under Experimental Procedures, and the samples were precipitated with ethanol. The figure shows an immunoblot probed with an affinity-purified CRD antiserum at a dilution of 1:500.

FIGURE 5: Carboxy terminus predicted for hamster PrP. The final lysine predicted from the nucleotide sequence is at residue 220. The GPI anchor is attached to the α-carboxy group of serine 231 as indicated by the arrow (N. Stahl et al., unpublished results). The synthetic peptide P3 spans the underlined residues.

released from the neuron-rich fraction, although substantial amounts of released PrP^C could be detected.

PIPLC Cleavage of Purified PrP 27–30. Although the appearance of anti-CRD immunoreactivity following PIPLC treatment of purified PrP 27–30 rods (Stahl et al., 1987) indicates at least partial susceptibility to the phospholipase, this does not provide a quantitative measure showing what fraction of the GPI anchors are cleaved. Traditional assays for phospholipase cleavage of GPI-anchored proteins take advantage of the change in hydrophobicity upon removal of lipid from native proteins as measured by Triton X-114 partitioning (Etges et al., 1986), binding to phenyl-Sepharose (Davitz et al., 1987b), or migration shifts in nondenaturing detergent gels (Helenius & Simons, 1977; Almqvist & Carlsson, 1988). However, the aggregation of PrP 27–30 rendered these assays unfeasible or questionable. We instead chose to investigate the intrinsic PIPLC susceptibility of a glycolipidated peptide derived from the carboxy terminus of PrP 27–30.

Examination of the PrP amino acid sequence predicted from the hamster cDNA and genomic open reading frames (Oesch et al., 1985; Basler et al., 1986) reveals that the final lysine residue in the molecule is 11 amino acids upstream of the site predicted (serine 231; Ferguson & Williams, 1988) for addition of the GPI anchor to PrP (Figure 5). We have recently mapped the attachment point of the GPI anchor for PrP 27–30; our results confirm this prediction (Stahl & Prusiner, 1989). Furthermore, this carboxy-terminal peptide contains two tyrosine residues, rendering it susceptible to iodination for easy detectibility. Thus, cleavage of radioiodinated PrP 27–30 with endoproteinase Lys-C is predicted to yield three radioactive peptides of 75, 16, and 11 amino acids (Oesch et al., 1985), the latter of which is attached to the GPI anchor.

Purified PrP 27–30 was denatured, solubilized, iodinated at its tyrosines, and incubated with endo Lys-C as described under Experimental Procedures. The products of the digest were subjected to electrophoresis on an SDS–polyacrylamide

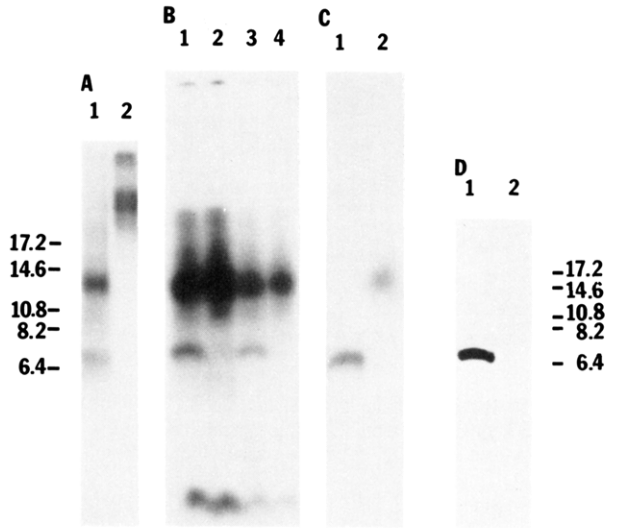

FIGURE 6: Endoproteinase Lys-C and PIPLC cleavage of PrP 27–30. This figure shows SDS–PAGE gels run with a tricine buffer system that allows separation of peptides. Radioiodinated PrP 27–30 was prepared and cleaved with endoproteinase Lys-C as described under Experimental Procedures. The gel in panel A was fixed and dried before autoradiography, while those in panels B and C were exposed to film immediately after electrophoresis. (A) PrP 27–30 after (lane 1) or before (lane 2) cleavage with endoproteinase Lys-C. (B) PIPLC treatment of ^{125}I-PrP 27–30 (lanes 2 and 4) was carried out either befeore (lanes 1 and 2) or after (lanes 3 and 4) digestion with endoproteinase Lys-C. (C) The carboxy-terminal peptide containing the GPI anchor was purified by HPLC and incubated without (lane 1) or with (lane 2) PIPLC before SDS–PAGE. (D) Immunoblot of endoproteinase Lys-C digested PrP 27–30 probed with an anti-P3 antiserum. This antiserum was raised against the synthetic peptide indicated in Figure 5. The sample in lane 2 was also incubated with PIPLC before electrophoresis.

gel with a tricine buffer system for the resolution of peptides (Schägger & von Jagow, 1987). An autoradiograph of this gel shows that PrP 27–30 (Figure 6A, lane 2) was digested completely to yield three radioactive fragments (Figure 6A,B, lane 1). The larger fragments migrate at 13 and 8 kDa, respectively, while the smallest band migrates near the solvent front (Figure 6B, lane 1) but more slowly than radioactive iodide. Preincubation of the digest with PIPLC results in the specific disappearance of the 8-kDa peptide without the appearance of a new band (Figure 6B, lanes 2 and 4).

Most GPI-anchored proteins, including PrP^C, migrate more slowly in SDS–PAGE after release of diradylglycerol by PIPLC (Low, 1987), and one might predict similar behavior for a glycolipidated peptide. Thus it seemed possible that the carboxy-terminal peptide comigrates with the largest fragment at 12 kDa after treatment with PIPLC.

In order to clarify this point, the carboxy-terminal endo Lys-C fragment of PrP 27–30 was purified by HPLC. Reverse-phase chromatography of the endo Lys-C digest of iodinated PrP 27–30 gives a radioactivity profile of two major peaks that follow a peak of radioactivity in the void volume (Figure 7). A small peak that elutes near the end of the gradient (fractions 80–83) corresponds to the PIPLC-sensitive carboxy-terminal fragment, which shifts to elute near the beginning of the gradient (fractions 32–34) when the sample is pretreated with PIPLC (Figure 7). We have consistently observed greater recovery of this peptide after removal of the covalent lipid with PIPLC. Incubation of this late-eluting peptide with PIPLC results in a large decrease in migration on the tricine–SDS gel system (Figure 6C), consistent with the above hypothesis.

Similar results are observed when nonradioactive PrP 27–30 is digested with endo Lys-C and immunoblotted with antisera

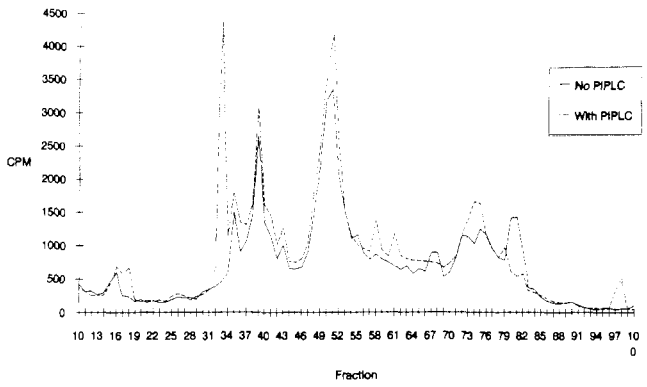

FIGURE 7: Reverse-phase HPLC of ^{125}I-PrP 27–30 after endoproteinase Lys-C digestion. The plot shows the counts per minute of ^{125}I in 1-min fractions collected across the gradient.

that was raised against a synthetic peptide called P3 (see Figure 5). One observes immunostaining of an 8-kDa band that disappears when the sample is pretreated with PIPLC (Figure 6D). It is likely that disappearance of the anti-P3 reactive band results from the inability of the small peptide to bind nitrocellulose or PVDF upon electrotransfer once the covalent lipid is removed.

Discussion

We have demonstrated in this report that PrP^C and PrP^{Sc} are differentially released from cellular membranes by PIPLC. While the majority of PrP^C is released upon cleavage of the GPI anchor with PIPLC, PrP^{Sc} remains cell associated after treatment with the phospholipase (Figure 1). Identical results were observed with cells isolated from hamsters both 45 and 65 days after inoculation with scrapie. Since there is no demonstrable pathology 45 days following scrapie infection in hamsters (Baringer et al., 1981), the differential release cannot be explained by some degenerative pathological process.

The nonrelease of PrP^{Sc} does not result from an intrinsic resistance of the glycolipid anchor to PIPLC. Figure 6 demonstrates that the GPI anchor attached to a carboxy-terminal peptide derived from purified PrP 27–30 is completely cleaved by PIPLC. This result is confirmed by other experiments in which >80% of the carboxy terminal peptide was recovered in a single HPLC peak following digestion of PrP 27–30 with endoproteinase Lys-C and PIPLC (N. Stahl et al., unpublished results). Thus the resistance of PrP^{Sc} to PIPLC release cannot be ascribed to a chemical modification of the glycolipid such as the palmitoylation of the inositol found in human erythrocyte acetylcholinesterase (Roberts et al., 1988).

The subcellular localization of PrP^{Sc} in dissociated brain cells remains uncertain, although the accessibility of PrP^{Sc} to proteinase K (Figure 2) and biotinylation with a membrane-impermeant reagent (Figure 3) implies that it should also be accessible to PIPLC. The observation that tubulin also becomes biotinylated suggests that the cells became leaky during the isolation procedure. Although we cannot conclude from these data that PrP^{Sc} is exposed on the cell surface in the isolated cells, it should be noted that treatment of minced brain chunks with proteinase K also caused conversion of PrP^{Sc} to PrP 27–30. Biotinylation of scrapie-infected cultures of mouse neuroblastoma cells indicates that PrP^{Sc} is localized in some interior compartment as well as the cell surface, although none is released by PIPLC (Borchelt et al., 1990). Indirect immunofluorescence of neuroblastoma cells with PrP antisera indicates that a major fraction of PrP^{Sc} is found in the cell interior localized with wheat germ agglutinin staining (Taraboulos et al., 1990).

Several possible explanations could account for the differential release of PrP^C and PrP^{Sc} by PIPLC.

First, although the amino terminus of PrP^{Sc} in the dissociated cells is accessible to proteinase K, it is possible that access to the carboxy-terminal glycolipid is restricted. This could result from self-association of PrP^{Sc} in the cell membrane. Purified PrP^{Sc} aggregates upon detergent extraction and forms amyloid rods after a limited digest with proteinase K removes the amino terminus to give PrP 27–30 (Meyer et al., 1986; McKinley et al., 1988). Purified PrP 27–30 in amyloid rods is at least partially susceptible to PIPLC (Stahl et al., 1987). While sonication of PrP 27–30 rods in the presence of phospholipid and detergent results in functional solubilization of the prion protein (Gabizon et al., 1987), the aggregation state of PrP^{Sc} in cellular membranes is unknown. There is precedent for this type of phenomenon: the VSG of *Trypanosoma brucei* cannot be released from cells by exogenous PIPLC (Cross, 1984) yet is cleavable following detergent lysis (Cardoso de Almeida & Turner, 1983). The inaccessibility of the GPI anchor of VSG to PIPLC is thought to result from tight packing of the VSG molecules on the cell surface (Cross, 1975; Tetley et al., 1981).

Second, access to the PrP^{Sc} glycolipid could be blocked through interaction of PrP^{Sc} with other cellular components. Several cellular proteins have recently been shown to bind radioiodinated PrP^{Sc} on ligand blots (Oesch & Prusiner, 1989). Whether PrP^{Sc} binds these proteins in cells remains to be determined. It is also feasible that the anchor *is* cleaved upon addition of PIPLC to the cells and PrP^{Sc} remains bound to a cellular component. Binding of Thy-1 to other cellular components (Hoessli & Rungger-Brändle, 1985) might account for the observations that half of the Thy-1 in thymocytes, fibroblasts, and lymphoma cells is relatively immobile (Ishihara et al., 1987) and half is resistant to release by PIPLC (Low, 1987). It is not known if the immobile portion of Thy-1 is PIPLC resistant and whether this fraction becomes PIPLC sensitive upon purification.

Third, it is conceivable that PrP^{Sc} has transmembrane peptide domains. In vitro translation of synthetic PrP mRNA made from a cDNA clone gives both a soluble (secreted) form and a form that crosses the membrane twice (Hay et al., 1987a,b). The first transmembrane domain appears to consist of hydrophobic amino acids, while the second is a region that is predicted to form an amphipathic helix (Bazan et al., 1987). Most of the PrP^C synthesized by cells is released by PIPLC and thus has no transmembrane domain. However, we cannot rule out the possibility that a small fraction of transmembrane PrP^C exists in cells. The membrane topology of PrP^{Sc} remains unknown. To our knowledge, no protein has been described to have both transmembrane peptide domains and a GPI anchor.

The biotinylation procedure that we have described provides a semiquantitative alternative to cell surface iodination. We chose the cleavable dithiol biotinylation reagent to eliminate the difficulty of dissociating biotin from streptavidin, which requires harsh conditions (Bayer & Wilchek, 1980). Estimation of the fraction of exposed protein by this procedure requires some important controls. Use of an internal biotinylated standard is strongly recommended to demonstrate complete retention of biotinylated proteins by the streptavidin–agarose. It is also advisable to determine that the modification of primary amino groups does not seriously diminish immunoreactivity of the protein. We have used both monoclonal and anti-peptide antibodies that show no diminution in binding to biotinylated PrP; some of our polyclonal anti-

bodies do show decreased binding.

Whether the differential release of PrP^C and PrP^{Sc} plays a role in scrapie pathogenesis is a matter of speculation. It is conceivable that the inability of PrP^{Sc} to be released by PIPLC is deleterious. A significant fraction of PrP^C is spontaneously released in a soluble form from *Xenopus* oocytes injected with PrP mRNA (Hay et al., 1987b) and a variety of cultured cells (Caughey et al., 1989; Borchelt et al., 1990). No spontaneous release of PrP^{Sc} from scrapie-infected neuroblastoma cells is observed (Borchelt et al., 1990). It is also unknown whether problems may result from the mislocalization of a portion of PrP^{Sc} to the cell interior, which has been demonstrated in the scrapie-infected neuroblastoma cells (Taraboulos et al., 1990; Borchelt et al., 1990). Although a variety of GPI-modified proteins are released normally by PIPLC in the scrapie-infected dissociated cells (Figure 4), we do not know if other GPI-anchored proteins accumulate during scrapie infection in a manner similar to that observed for PrP^{Sc}. A recent report demonstrates that addition of a GPI anchor to H-2 protein in T lymphocytes is sufficient to give activation of the cells upon cross-linking with anti-H-2 antibodies (Robinson et al., 1989). Although the pathway for this signal is unknown, it is conceivable that the aggregation of GPI-anchored PrP^{Sc} may result in deleterious interaction with an analogous signaling pathway in brain cells.

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REFERENCES

Almqvist, P., & Carlsson, S. R. (1988) *J. Biol. Chem. 263*, 12709–12715.

Alper, T., Cramp, W. A., Haig, D. A., & Clarke, M. C. (1967) *Nature (London) 214*, 764–766.

Barbet, A. F., & McGuire, T. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1989–1993.

Baringer, J. R., Prusiner, S. B., & Wong, J. S. (1981) *J. Neuropathol. Exp. Neurol. 40*, 281–288.

Barry, R. A., & Prusiner, S. B. (1986) *J. Infect. Dis. 154*, 518–521.

Barry, R. A., McKinley, M. P., Bendheim, P. E., Lewis, G. K., DeArmond, S. J., & Prusiner, S. B. (1985) *J. Immunol. 135*, 603–613.

Barry, R. A., Kent, S. B., McKinley, M. P., Meyer, R. K., DeArmond, S. J., Hood, L. E., & Prusiner, S. B. (1986) *J. Infect Dis. 153*, 848–854.

Barry, R. A., Vincent, M. T., Kent, S. B. H., Hood, L. E., & Prusiner, S. B. (1988) *J. Immunol. 140*, 1188–1193.

Basler, K., Oesch, B., Scott, M., Westaway, D., Wälchli, M., Groth, D. F., McKinley, M. P., Prusiner, S. B., & Weissmann, C. (1986) *Cell 46*, 417–428.

Bayer, E. A., & Wilchek, M. (1980) *Methods Biochem. Anal. 26*, 1–45.

Bazan, J. F., Fletterick, R. J., McKinley, M. P., & Prusiner, S. B. (1987) *Protein Eng. 1*, 125–135.

Bellinger-Kawahara, C., Cleaver, J. E., Diener, T. O., & Prusiner, S. B. (1987a) *J. Virol. 61*, 159–166.

Bellinger-Kawahara, C. G., Diener, T. O., McKinley, M. P., Groth, D. F., Smith, D. R., & Prusiner, S. B. (1987b) *Virology 160*, 271–274.

Bellinger-Kawahara, C. G., Kempner, E., Groth, D., Gabizon, R., & Prusiner, S. B. (1988) *Virology 164*, 537–541.

Bendheim, P. E., Potempska, A., Kascsak, R. J., & Bolton, D. C. (1988) *J. Infect. Dis. 158*, 1198–1208.

Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1982) *Science 218*, 1309–1311.

Bolton, D. C., McKinley, M. P., & Prusiner, S. B. (1984) *Biochemistry 23*, 5898–5906.

Bolton, D. C., Meyer, R. K., & Prusiner, S. B. (1985) *J. Virol. 53*, 596–606.

Borchelt, D. R., Scott, M., Taraboulos, A., Stahl, N., & Prusiner, S. B. (1990) *J. Cell Biol. 110*, 743–752.

Butler, D. A., Scott, M. R., Bockman, J. M., Borchelt, D. R., Taraboulos, A., Hsiao, K. K., Kingsbury, D. T., & Prusiner, S. B. (1988) *J. Virol. 62*, 1558–1564.

Cardoso de Almeida, M. L., & Turner, M. J. (1983) *Nature 302*, 349–352.

Caughey, B., Race, R. E., Ernst, D., Buchmeier, M. J., & Chesebro, B. (1989) *J. Virol. 63*, 175–181.

Chao, S.-W., & Rumsby, M. G. (1977) *Brain Res. 124*, 347–351.

Cho, H. J. (1986) *J. Gen. Virol. 67*, 243–253.

Cross, G. A. M. (1975) *Parasitology 71*, 393–417.

Cross, G. A. M. (1979) *Nature 277*, 310–312.

Cross, G. A. M. (1984) *J. Cell. Biochem. 24*, 79–90.

Davitz, M. A., Gurnett, A. M., Low, M. G., Turner, M. J., & Nussenzweig, V. (1987a) *J. Immunol. 138*, 520–523.

Davitz, M. A., Hereld, D., Shak, S., Krakow, J., Englund, P. T., & Nussenzweig, V. (1987b) *Science 238*, 81–84.

Diener, T. O., McKinley, M. P., & Prusiner, S. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5220–5224.

Diringer, H., Gelderblom, H., Hilmert, H., Ozel, M., Edelbluth, C., & Kimberlin, R. H. (1983) *Nature 306*, 476–478.

Endo, T., Groth, D., Prusiner, S. B., & Kobata, A. (1989) *Biochemistry 28*, 8380–8388.

Etges, R., Bouvier, J., & Bordier, C. (1986) *J. Biol. Chem. 261*, 9098–9101.

Ferguson, M. A. H., & Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285–320.

Gabizon, R., McKinley, M. P., & Prusiner, S. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4017–4021.

Gabizon, R., McKinley, M. P., Groth, D. F., Kenaga, L., & Prusiner, S. B. (1988a) *J. Biol. Chem. 263*, 4950–4955.

Gabizon, R., McKinley, M. P., Groth, D., & Prusiner, S. B. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6617–6621.

Haraguchi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarantino, A., Teplow, D., Hood, L., Burlingame, A., Lycke, E., Kobata, A., & Prusiner, S. B. (1989) *Arch. Biochem. Biophys. 274*, 1–13.

Hay, B., Barry, R. A., Lieberburg, I., Prusiner, S. B., & Lingappa, V. R. (1987a) *Mol. Cell. Biol. 7*, 914–920.

Hay, B., Prusiner, S. B., & Lingappa, V. R. (1987b) *Biochemistry 26*, 8110–8115.

Helenius, A., & Simons, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 529–532.

Hoessli, D., & Rungger-Brändle, E. (1985) *Exp. Cell Res. 156*, 239–250.

Holder, A. A., & Cross, G. A. M. (1981) *Mol. Biochem. Parasitol. 2*, 135–150.

Hope, J., Morton, L. J. D., Farquhar, C. F., Multhaup, G., Beyreuther, K., & Kimberlin, R. H. (1986) *EMBO J. 5*, 2591–2597.

Hope, J., Multhaup, G., Reekie, L. J. D., Kimberlin, R. H., & Beyreuther, K. (1988) *Eur. J. Biochem. 172*, 271–277.

Hunter, G. D. (1979) in *Slow Transmissible Diseases of the Nervous System* (Prusiner, S. B., & Hadlow, W. J., Eds.) Vol. 2, pp 365–385, Academic Press, New York.
Ishihara, A., Hou, Y., & Jacobson, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1290–1293.
Kingsbury, D. T., Smeltzer, D., & Bockman, J. (1984) *Sixth International Congress on Virology*, Sendai, Japan, p 70 (Abstract).
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Low, M. G. (1987) *Biochem. J. 244*, 1–13.
Manuelidis, L., Sklaviadis, T., & Manuelidis, E. E. (1987) *EMBO J. 6*, 341–347.
McKinley, M. P., Bolton, D. C., & Prusiner, S. B. (1983a) *Cell 35*, 57–62.
McKinley, M. P., Masiarz, F. R., Isaacs, S. T., Hearst, J. E., & Prusiner, S. B. (1983b) *Photochem. Photobiol. 37*, 539–545.
McKinley, M. P., Meyer, R., Kenaga, L., Rahbar, F., Serban, A., Gabizon, R., & Prusiner, S. B. (1988) *J. Cell Biol. 107*, 725a (Abstract).
Meyer, R. K., McKinley, M. P., Bowman, K. A., Braunfeld, M. B., Barry, R. A., & Prusiner, S. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2310–2314.
Norton, W. T., & Poduslo, S. E. (1970) *Science 167*, 1144–1146.
Oesch, B., & Prusiner, S. B. (1989) *Clin. Res. 37*, 566A (Abstract).
Oesch, B., Westaway, D., Wälchli, M., McKinley, M. P., Kent, S. B. H., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B., & Weissmann, C. (1985) *Cell 40*, 735–746.
Prusiner, S. B. (1982) *Science 216*, 136–144.
Prusiner, S. B. (1989) *Annu. Rev. Microbiol. 43*, 345–374.
Prusiner, S. B., Bolton, D. C., Groth, D. F., Bowman, D. A., Cochran, S. P., & McKinley, M. P. (1982) *Biochemistry 21*, 6942–6950.
Prusiner, S. B., McKinley, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., & Glenner, G. G. (1983) *Cell 35*, 349–358.
Prusiner, S. B., Groth, D. F., Bolton, D. C., Kent, S. B., & Hood, L. E. (1984) *Cell 38*, 127–134.
Race, R. E., Fadness, L. H., & Chesebro, B. (1987) *J. Gen. Virol. 68*, 1391–1399.
Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G., & Rosenberry, T. L. (1988) *J. Biol. Chem. 263*, 18766–18775.
Robinson, P. J., Millrain, M., Antoniou, J., Simpson, E., & Mellor, A. L. (1989) *Nature 342*, 85–87.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Stahl, N., & Prusiner, S. B. (1989) *Protein Soc.*, S160 (Abstract).
Stahl, N., Borchelt, D. R., Hsiao, K., & Prusiner, S. B. (1987) *Cell 51*, 229–240.
Stieger, A., Cardoso del Almeida, M.-L., Blatter, M.-C., Brodbeck, U., & Bordier, C. (1986) *FEBS Lett. 199*, 182–186.
Taraboulos, A., Serban, D., & Prusiner, S. B. (1990) *J. Cell Biol.* (in press).
Tetley, L., Vickerman, K., & Moloo, S. K. (1981) *Trans. R. Soc. Trop. Med. Hyg. 75*, 409–414.
Turk, E., Teplow, D. B., Hood, L. E., & Prusiner, S. B. (1988) *Eur. J. Biochem. 176*, 21–30.